REMARKS

Claims 15-17, 20, 21, 23, 24 and 25-46 were pending in the case. Upon entry of this Amendment, the pending claims will be cancelled and will be replaced with Claims 47-56.

The Office Action raises an enablement rejection with regard to the use of VLCFAE polypeptides (or "KAS") which are not the polypeptide of Seq. ID. No. 2 and argues that it is not possible for a person skilled in the art to decide which VLCFAE has to be used in the claimed method.

Applicants respectfully traverse, but to remove issues and advance prosecution, the claims have now been restricted to the use of a polypeptide with an identity of at least 90% to said sequence (Claims 47, 48, 51 and 52), and to the use of a polypeptide with an identify of at least 90%, (Claims 49, 50, 53, and 54). Support for this Amendment may be found in the specification at page 6, line 3, among other places.

The Examiner raises a question of guidance to one skilled in the art for the use of polypeptides which are not identical with SEQ ID No. 2. Applicants respectfully point out that VLCFAE's are 3-ketoacyl-CoA-synthases (KAS). The polypeptide of SEQ ID No. 2 is such an enzyme as are the ones disclosed in U.S. Patent No. 6,307,128 and Accession No. 064846, as noted by the Examiner. The present invention teaches that such 3-ketoacyl-CoA-synthases can be used as targets to identify herbicides because they are susceptible to small compounds and because the inhibition of their enzymatic activity leads to death or severe damage of a target plant. Such enzymes have not been heretofore known as targets for herbicides. VLCFAE's are known enzymes (e.g. U.S. Patent No. 6,307,128 and Acc. No. 064846) and can be easily identified by a person of skill in the art.

Therefore, the polypeptide of SEQ ID NO. 2 has been used as an example to demonstrate that the claimed method achieves its desired result. The contribution of the present invention to the art therefore is not that only the specific polypeptide of SEQ. ID NO. 2 is a target, but that plant polypeptides having the enzymatic activity of a 3-ketoacyl-CoA-synthase are targets for herbicidally active compounds and can thus be effectively used in the claimed method for identifying new herbicidal

compounds that were not known to have herbicidal activity. Given the contribution of the present invention to the art, it would not be fair to the inventors to limit the present invention merely to SEQ ID NO. 2.

Further, the specification clearly states that VLCFAEs show extensive homologies with one another (please see page 3, line 15 of the specification). And, therefore it is possible to use homologous polypeptides for the same purpose. Examples for such 3-ketoacyl-CoA-synthases are explicitly given in Table 1 on page 4 of the specification. The specification further teaches that in particular, polypeptides which are homologous to FDS (SEQ ID NO. 2) can be used, particularly those with an identity of at least 90% (please see pages 4-5 of the specification).

Applicants respectfully urge that it is the general state of the art to identify on the basis of a given DNA or polypeptide sequence (SEQ ID NO:1, encoding SEQ ID NO:2) other DNA sequences or polypeptide sequences which are homologous. This can easily be done, for example, by

a) searching databases for homologous sequences (preferably based on amino acid sequences) with a sensitive sequence comparison algorithm,

b) PCR with oligonucleotides derived from the starting sequence (SEQ ID NO:1),

c) hybridization assays with adequate stringency.

No explicit guidance is needed for a person with skill in the art to obtain a polypeptide which - based on sequence homology - is at least 90% homologous to SEQ ID NO:2.

The question then becomes whether the homologous polypeptide sequences will have the same enzymatic activity and function. In this case, Applicants respectfully urge that the claims are directed to 90% homology. Applicants respectfully urge that it is now generally accepted in the scientific community, that at a sequence identity of 25% or greater over an alignment length of at least 80 amino acids, a structural similarity between two polypeptides will be established, and a

identity of above 40%. Polypeptides having an identity of at least **90%** are generally referred to as being extremely similar or "basically identical" in structure and function. Applicants include herewith two references for the Examiner's consideration. The first is a plot of the Relationship of Similarity in Sequence (**Attachment A)** and the second is an article entitled "Structural Proteomics As A Tool For Functional Assignment And Target Validation," Sundstrom M., Business Briefing: Future Drug Discovery (**Attachment B)** (see underlined paragraphs therein).

A person skilled in the art, having determined (e.g. by a database search) additional polypeptides having an identity of at least 90% to SEQ ID NO:2 would have no doubts that the enzymatic activity and function of the polypeptide of SEQ ID NO:2 found is identical to the one described for SEQ ID NO:2. U.S. Patent No. 6,306,128 only confirms that polypeptides with a high homology to SEQ ID NO:2 (see statements of the Examiner as to the homology of the sequences) would have the same function. In this reference, which is cited in the specification as WO 98/54954, it is shown very clearly that the sequences with homology to SEQ ID NO:2 have a 3-ketoacyl-CoA-synthase activity.

Additional guidance is given in the specification at page 15, line 20, where it is clearly stated that "the first 45-50 amino acids at the N-terminus are specific" for the FDH protein and can be used as a recognition sequence. The amino acids referred to are part of the sequence listing. A person with skill in the art is thus taught how to check on the identity of any polypeptide he or she has identified as having an identity of at least 90% by comparing the respective N-termini.

Applicants call to the Examiner's attention that the specification explicitly refers to WO 98/54954 (see page 1, line 18), wherein an assay for testing the enzymatic activity of 3-ketoacyl-CoA-synthases are described (see Example 2, page 19, of the reference). Assays for assessing the enzymatic activity of 3-ketoacyl-CoA-synthases (VLCFAEs) were thus well known in the art at the time the present application was filed, (see for example, also Hlousek-Radojcic et al. (1998), attached hereto at **Attachment C,** where another assay is described). A person

skilled in the art would therefore be enabled to additionally check the identity of any polypeptide he or she has identified as being at least 90% identical to SEQ ID NO:2 by performing an activity assay according to the assays known in the art.

In conclusion, Applicants respectfully urge that a person with skill in the art is enabled by what is taught in the application to identify and use in the claimed method a polypeptide according to the invention, having an identity of at least 90% to SEQ ID NO: 2. The Wand factors mentioned by the Examiner on page 4 of the Office Action can be answered in a way that based on the present scope of the claims not undue experimentation is needed to perform the method claimed, the claimed method does not contain any unpredictabilities and that the claims are sufficiently clear and enabled by the specification to permit one skilled in the art to use the present invention.

Applicants believe the newly added claims overcome the rejections set forth on page 7 of the Office Action, and Applicants respectfully request withdrawal of the 35 U.S.C. Section 112 (second paragraph) rejection.

The Office Action alleged the prior claims were anticipated under 35 U.S.C. Section 102 as being anticipated by U.S. Patent No. 6,306,128 to Jorwarski et al (hereinafter "Jorwarski"). Jorwarski describes nucleic acids and polypeptides having the enzymatic activity of 3-ketoacyl-CoA-synthases and methods of using them to produce **transgenic plants with an altered level of said enzymes**.

The present claims are directed to an entirely different invention. They are directed to **methods for identifying herbicides**. Jorwarski also describes the influence of various cofactors and small molecules like cerulenin on the enzymatic activity of the KAS. However, Jorwarski teaches only which cofactors should be used in order to achieve an optimal enzymatic activity of KAS (VLCFAE). Jorwarski **importantly, fail to teach that the inhibition of plant KAS by small molecules leads to the death or damage of a plant, and that herbicides can be identified by performing activity assays with said polypeptides.** Jorwarski do not teach methods for identifying herbicides, therefore it cannot anticipate the present invention.

The Office Action also rejected the claims for reasons of obviousness over U.S. Patent No. 6,306,128 and Acc. No. 064,846. The Office Action alleges that these references teach nucleic acids being highly similar or identical to SEQ ID NO:1.

However, no nucleic acids or polypeptides are claimed in the present application. The invention claimed is a method for identifying herbicides, and neither reference contains any suggestion or teaching directed to the claimed invention.

The high similarity of the sequences mentioned in the reference to the one mentioned in the present invention is therefore not relevant as long as the reference does not teach that the sequences mentioned therein are targets for herbicides and can be used **in methods for identifying new herbicidally active compounds**.

It is said in the Office Action that U.S. Patent No. 6,306,128 teaches methods for evaluating the influence of difference cofactors or small molecules such as cerulenin on the enzymatic activity of VLCFAEs (KAS). However, it is important to look at what the reference teaches in the context that it is taught. The reference teaches only that the enzymatic activity can be influenced depending upon the cofactor present in the activity assay and that, for example, cerulenin, an antifungal antibiotic isolated from *Cephalosporium caerulens,* does not influence the enzymatic activity of the KAS tested (see column 18, line 25). The reference doe not include the slightest indication, hint, teaching or suggestion that an inhibitor of VLCFAEs could be used as a herbicide. The reference does not even suggest that VLCFAEs are essential enzymes for the viability of plants. Therefore, the disclosure of U.S. Patent No. 6,306,128 does not suggest or motivate a person with skill in the art to use VLCFAE in methods for identifying herbicides. WO 064,846 does not offer any disclosure which closes the gap between U.S. Patent No. 6,306,128 and the presently claimed invention, as it only discloses another sequence information.

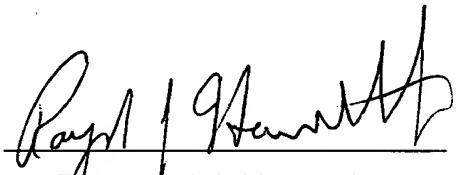
Further, while Jorwarski suggests to regulate the VLCFAE activity in a plant, there is no suggestion that this regulation is connected with identifying a herbicide or test/predicting herbicidal activity. In fact, by suggesting that the regulation of KAS in a plant can be used for "increased seed germination percentage, increasing seedling

vigor, increased resistance to seedling fungal diseases" (column 8, line 50), the disclosure of Jorwarski clearly **teaches away** from the present invention. Applicants therefore respectfully urge that the present claims are not rendered obvious over these references, either alone or in combination with one another.

Applicants believe the foregoing addresses all issues raised in the Office Action. Review and consideration of the claims and allowance thereof are respectfully requested.

If the Examiner believes issues remain, the Examiner is invited to telephone the undersigned and discuss those issues before issuing another Office Action in the case.

Respectfully submitted,

By 

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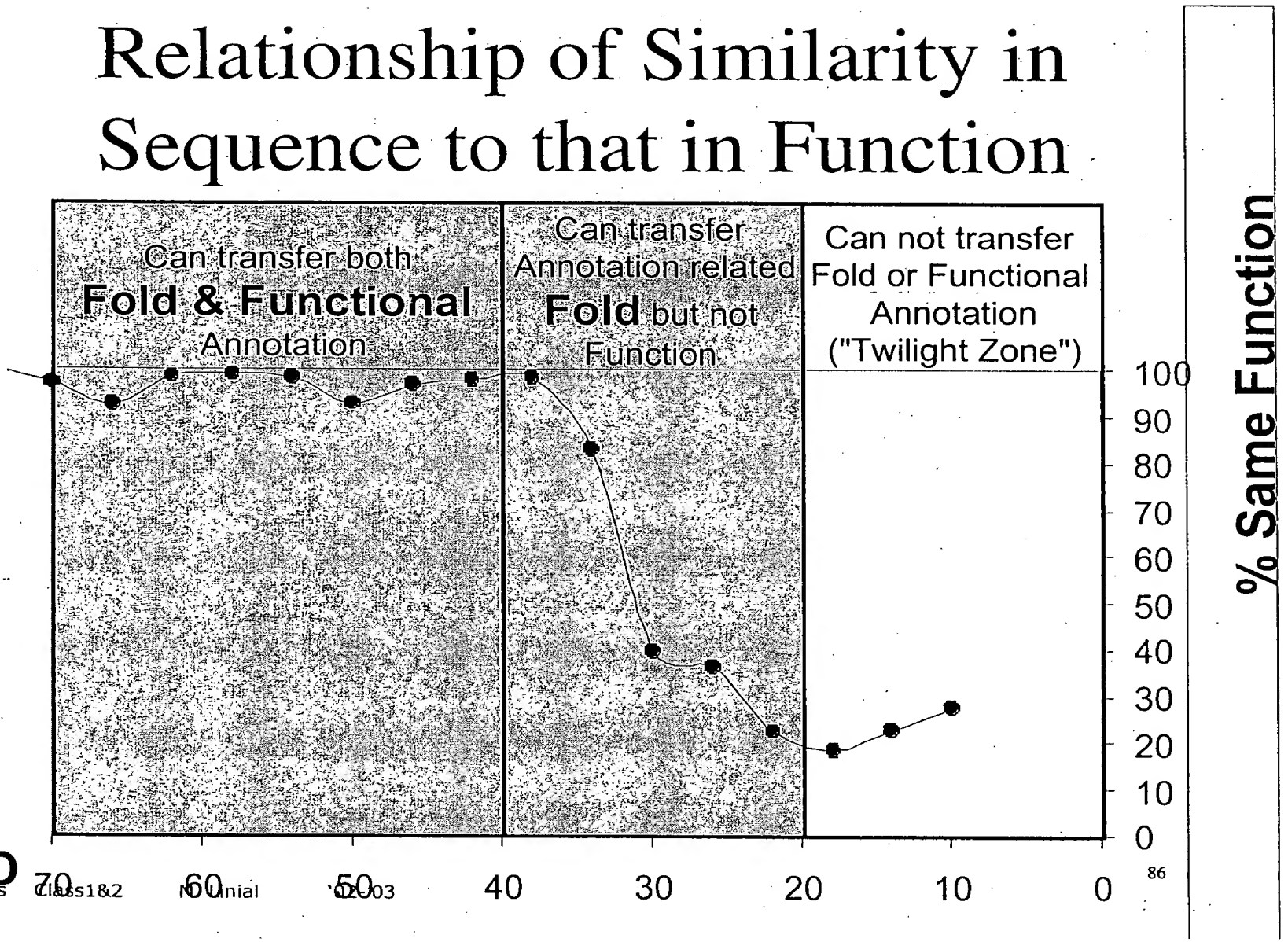
Relationship of Similarity in Sequence to that in Function



ATTACHMENT A

Structural Proteomics as a Tool for Functional Assignment and Target Validation

a report by
Michael Sundström

Former Chairman, Oncology Research Review Committee, and Vice President, Discovery Research, Actar AB

Introduction

Proteomics has recently become an indispensable tool for the high-throughput identification and analysis of expressed proteins from cells – the proteome. Particular interest has been given to the study of their expression levels as a consequence of the developmental stage or external stimuli such as drugs. Proteomics methods generate more 'detailed' knowledge compared with analysis of deoxyribonucleic acid (DNA) sequences because, for example, it is possible to identify post-translational modifications and interaction components that modulate the activity of the target.

Particularly significant and interesting approaches have arisen within numerous commercial and academic structural proteomics initiatives. The research discipline emerged as a consequence of development and implementation of high-throughput methodologies and technologies that enable novel data to be generated with speed and accuracy regarding:

- protein production – using high-throughput cloning and expression from multiple vectors in multiple hosts, core domain identification using proteolysis methods and use of expression and detection tags;
- protein crystallography – this has improved significantly when freezing of crystals at liquid nitrogen temperature (cryofreezing), multiple-wavelength anomalous dispersion phasing, robotisation, automated data collection and the use of synchrotron beamlines were adopted as standards; and
- biomolecular nuclear magnetic resonance (NMR) – for which improvements in structure determination using isotope-enriched protein samples, access to high field spectroscopy and cryogenic probes as well as automated spectra assignment and structure determination have greatly improved its usefulness.

Despite major improvements regarding the ability to carry out true high-throughput structure determination and prediction, two major limiting factors are easily identifiable:

- efficient and rational production of "biologically relevant" proteins with desired physical properties; and
- the ability to correctly model structures using low-sequence homology templates.

Key Methodology and Technology Advances

From Sequence to Function

The most straightforward approaches to predicting protein function are independent of any direct information of protein conformation. Here, DNA, or protein amino-acid sequences, is used as input data and is the preferred choice for automatic annotations of large DNA sequence data sets and entire genomes. In recent reports on the sequencing analysis of the human genome, it was shown that between 40% and 60% of the predicted genes could be computationally assigned to functional categories. Although sequence comparison methods have improved, it is likely that a large portion of the potential genes will remain unpredicted.

Structure Prediction

Prediction methods of protein conformation can be divided into three major approaches:

- comparative/homology modelling;
- fold recognition/threading; and
- *ab initio* methods.

The most straightforward way to predict the conformation of a protein is to use a closely related (>30% sequence identity) high-quality experimental structure as a template, and align the query sequence to the sequence of a template structure. Such methods are termed 'homology' or 'comparative' modelling. The key step in such approaches is the correct structural alignment of the query sequence to the template. If the alignment is incorrect there are no model refinement methods available to compensate for such errors.

In threading algorithms, the fold is predicted by assessing the compatibility of a sequence to particular substructures of a template by analysing the intramolecular distances as the sequence is threaded through a collection of known folds. The environments of the amino-acid residues are assessed using knowledge-based scoring functions. Such methods can, in some cases, assign the correct fold for very distant sequence relationships.

The term '*ab initio* modelling of protein conformation' refers to methods that use the primary amino-acid sequence as the single input for predicting the structure. Thus, *ab initio* methods can be applied when related proteins with known folds cannot be identified. Currently, the *ab initio* models are unrefined, resulting in the best cases with deviations of 4Å root mean square deviation to the native structure. At the current stage of development it is questionable whether *ab initio* models can add significant information about the specific functions of novel genes.

Structural Templates

Structural proteomics aims to generate useful three-dimensional (3-D) structures of entire proteomes by a combination of experimental structure determination and modelling. However the limited number of available 3-D protein structure templates currently restricts attempts to make large-scale functional annotation using structural information. Depending on the organism, it has been assessed that 20% to 70% of individual proteomes can be modelled at the 30% sequence identity threshold level.

An attempt was made recently to estimate the number of structures needed to model 90% of the sequences, at >30% sequence identity, of the currently known protein families.[1] Based on available genome data and unique structures from the Research Collaboratory for Structural Bioinformatics' (RCSB's) Protein Data Bank (PDB), it was shown that structural templates were available for segments of 30% to 35% of the genome sequences, but it was also predicted that <10% of the residues could be modelled. Considering the predicted 'global' proteome, the estimate was made that ~16,000 novel and optimally selected structures would need to be generated to be able to model 90% of the sequences.

Structural proteomics approaches have fundamentally changed the structural biology research area and rapidly substantiate knowledge of protein folds at an unprecedented rate.

Protein Production

The success of high-throughput structure determination and subsequent structural analysis relies heavily on high-throughput protein production of suitable quality, as the physical properties of expressed proteins do not always allow a successful structure determination. The most critical bottlenecks reported are solubility and aggregation state, but other crucial factors include the availability of methods for rapid and accurate analysis of purity, homogeneity and structural integrity.

For structural proteomics efforts, at least in this initial phase, target proteins should be selected that, with minimum effort, are easy to express with the appropriate characteristics and give good quality NMR spectra or form-diffracting crystals, as most proteins that will be structurally determined are likely to be of significant value. The risk with this approach is that certain folds could become over-represented by time and other target types will not appear until a directed effort is attempted.

Although the use of expression and detection tags is a prerequisite for high-throughput approaches, it can be hypothesised that larger fusion partners can give misleading data by solubilising poorly behaving expression constructs. Thus, optimisation for the best fusion-tag-using solubility screens needs to be accompanied by quality analysis of the protein to assure that the constructs chosen for further studies are folded correctly and biologically relevant/active.

By default, the function of the protein is most often unknown in structural proteomics projects. However, as our knowledge increases, functional testing of the expressed proteins using directed assays – suggested by the structural features – should be adopted as standard.

Michael Sundström was appointed at the Center for Genomics and Bioinformatics, Karolinska Institute up to 2001 and, from 1998 to 2000, served as Chairman for the Oncology Research Review Committee as well as Director of Structural Chemistry and Informatics at Pharmacia's Discovery Research Oncology Unit in Milan. Following postdoctoral training at the Center for Structural Biochemistry at the Karolinska Institute, he joined the Pharmacia Discovery Research site in Stockholm as scientist and subsequently headed Protein Crystallography, as well as being Project Leader in Drug Discovery. He joined Actar AB as Vice President of Discovery Research in 2001. Mr Sundström earned his PhD from the Department of Molecular Biology at the Uppsala Biomedical Center in 1992.

1. *D Vitkup, et al., "Completeness in Structural Genomics"*, Natural Structural Biology, *8 (2001), pp. 559–566.*

ATTACHMENT B

cular Nuclear Magnetic
ce (NMR)

state NMR has emerged to play a key role
ural proteomics projects and has already
insights into structure-function
ips for a large number of proteins
current methodologies do not allow NMR
bed for structural investigations on full
due to protein size limitations (typically
dues), it has been estimated that between
5% of proteomes will be suitable for NMR
nce such a fraction of any given proteome
a wealth of novel targets for structural
potentially more serious limitation for
key structural proteomics methodology is
sample property requirements needed,
lude high solubility in aqueous solutions
ty in solution over extended time periods
a collection

Protein Crystallography

High-throughput crystallography has been facilitated by improved phasing and model-building methods, reduced amounts of sample required through miniaturisation and automation from the crystallisation stage to structure determination. Examples can be provided of those developing high-throughput capabilities for protein expression, crystallisation, image analysis for automatic crystal detection and structure determination.[2]

Structure – Function Prediction

Proteins sharing the same fold can have quite different functions. Recent studies conclude that precise function seems to be conserved down to 40% sequence identity, whereas a broader definition of a functional class is conserved down to 25% to 30% identity.[3,4]

s include Integrative Proteomics™ (http //www integrativeproteomics.com), Structural GenomiX www stromix com) and Syrrx, Inc (http //www syrrx com)

i, et al, "Assessing Annotation Transfer for Genomics: Quantifying the Relations Between Protein e, Structure and Function Through Traditional and Probabilistic Scores", Journal of Molecular Biology, *00), pp 233–249*

, et al, "Evolution of Function in Protein Superfamilies, from a Structural Perspective", Journal of lar Biology, *307 (2001), pp 1,113–1,143*

With increased coverage of protein 3-D structural space, it is expected that evolutionary relationships and functional assignment of single proteins and families will be greatly facilitated. In a limited, but significant, number of cases, direct electron density for 'native' ligands or co-factors bound to the protein has been observed in structures derived from X-ray crystallography. When such data is available at high resolution, hypothesis generation on the function of the protein can often be relatively straightforward.

Although a few studies on structure-based assignment of single proteins from experimental structures have emerged, the structural proteomics effort on the *archaeon Methanobacterium thermo-autotrophicum* represent the best published case study.[5] Here, the equivalent of 25% of the organism's proteome (1,871 open reading frames) were chosen for high-throughput structure determination and subsequent functional assignment.

Approximately 20% of the target proteins were found to be suitable candidates for structure determination. Further, the study revealed that poor expression and solubility of the proteins accounted for close to 60% of the failures and that NMR data collection and crystallisation were the two major time and resource consumers in the process. Ten structures derived using NMR and X-ray were published simultaneously. Five out of 10 structures contained a bound ligand or a ligand-binding site that could be inferred from structural homology. Thus, many of the structures suggested several functional assays that could be used to provide insights of function.

Functional epitopes and active sites in proteins are built from a limited number of amino-acid residues in precise 3-D relative positions. The PROSITE database of protein families and domains contains sequence patterns of functional sites, but has no 3-D information. The features of the PROSITE patterns have been analysed to define structural templates for structure-based mining for functional sites.[6] In another approach applied to model-built structures, 'Fuzzy Functional Forms', based on intramolecular distances between amino-acid residues were established.[7] However, at present, very few cases have been reported where proposed functions have been confirmed experimentally.

Conclusions

Structural proteomics approaches have fundamentally changed the structural biology research area and rapidly substantiate knowledge of protein folds at an unprecedented rate. It is also likely that directed structural proteomics efforts within larger protein families already characterised for function will impact with immediate effect on academic and industrial research. Especially important will be the generation of data based on 3-D structural features to understand determinants that drive specificity of inhibitors and antagonists.

A key factor for long-term success in structural proteomics is the establishment of efficient protein production systems, which gives biologically relevant constructs and an ability to probe a wide array of target types, including 'more difficult' targets such as protein complexes, enzymes requiring post-translational modifications for activity and, obviously, membrane-integrated targets.

Structure determination and analysis will not necessarily mean that function can be derived. Structures will, however, allow directed efforts in terms of validating a hypothetically assigned function using functional assays suggested by the structural topology and identified structural motifs.

Structure prediction methods will be a crucial tool for the generation of full 3-D structure maps. As the number of structural templates increases, a vast experimental pool of data will be available for the optimisation and improvement of theoretical approaches and hopefully lead to a rapid improvement in the accuracy and speed of such efforts.

The understanding of structural features for target proteins should add significant value to later stages of drug development in which structural and clinical genomics data could be combined to generate structurally annotated single nucleotide polymorphism maps. Such data has the potential to play a crucial role in the prediction of patient drug response as well as for the development of inhibitors and antagonists with optimal chance of being active against as large a pool of patients as possible. ■

5. D Christendat, et al., "Structural Proteomics of an Archaeon", Natural Structural Biology, *7 (2000), pp. 903–909.*

6. A Kasuya and J Thornton, "Three-dimensional Structure Analysis of PROSITE Patterns", Journal of Molecular Biology, *286 (1999), pp. 1,673–1,691.*

7. J Fetrow, et al., "Functional Analysis of the Escherichia coli *Genome Using the Sequence-to-structure-to-function Paradigm: Identification of Proteins Exhibiting the Glutaredoxin/thioredoxin Disulfide Oxidoreductase Activity"*, Journal of Molecular Biology, *281 (1998), pp. 949–968.*

Plant Physiol. (1998) 116: 251–258

Fatty Acid Elongation Is Independent of Acyl-Coenzyme A Synthetase Activities in Leek and *Brassica napus*[1]

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In both animal and plant acyl elongation systems, it has been proposed that fatty acids are first activated to acyl-coenzyme A (CoA) before their elongation, and that the ATP dependence of fatty acid elongation is evidence of acyl-CoA synthetase involvement. However, because CoA is not supplied in standard fatty acid elongation assays, it is not clear if CoA-dependent acyl-CoA synthetase activity can provide levels of acyl-CoAs necessary to support typical rates of fatty acid elongation. Therefore, we examined the role of acyl-CoA synthetase in providing the primer for acyl elongation in leek (*Allium porrum* L.) epidermal microsomes and *Brassica napus* L. cv Reston oil bodies. As presented here, fatty acid elongation was independent of CoA and proceeded at maximum rates with CoA-free preparations of malonyl-CoA. We also showed that stearic acid ([1-^{14}C]18:0)-CoA was synthesized from [1-^{14}C]18:0 in the presence of CoA-free malonyl-CoA or acetyl-CoA, and that [1-^{14}C]18:0-CoA synthesis under these conditions was ATP dependent. Furthermore, the appearance of [1-^{14}C]18:0 in the acyl-CoA fraction was simultaneous with its appearance in phosphatidylcholine. These data, together with the results of a previous study (A. Hlousek-Radojcic, H. Imai, J.G. Jaworski [1995] Plant J 8: 803–809) showing that exogenous [^{14}C]acyl-CoAs are diluted by a relatively large endogenous pool before they are elongated, strongly indicated that acyl-CoA synthetase did not play a direct role in fatty acid elongation, and that phosphatidylcholine or another glycerolipid was a more likely source of elongation primers than acyl-CoAs.

Very-long-chain fatty acids (>18 carbons) are found in the glucocerebrosides of plant plasma membranes (Cahoon and Lynch, 1991), in the storage lipids of many oil seed species (Harwood, 1980), and as precursors of plant epicuzymes of acyl elongation are membrane bound and generally thought to be associated with the ER and possibly the plasma membranes in vegetative cells (von Wettstein-Knowles, 1993) or with the ER (Agrawal et al., 1984) and oil bodies of developing seeds (Imai et al., 1995). Furthermore, the intermediates of acyl elongation are esterified to CoA rather than to the acyl carrier protein cofactor of fatty acid synthase (Fehling and Mukherjee, 1991).

Studies on acyl elongation in plants and animals indicate that, in general, the properties of acyl elongation in plants and animals are quite similar (for review, see Cinti et al., 1992; Cassagne et al., 1994). Both plant and animal elongases require malonyl-CoA and NAD(P)H and use a variety of primers with varied requirements for ATP. For example, relatively high rates of acyl elongation can be achieved without supplied primer, i.e. by ATP-dependent elongation of endogenous substrates. This implies that there is a sufficient endogenous primer pool, but that ATP is necessary to use it.

The extent of the ATP requirement for elongation of exogenous acyl-CoAs apparently depends on the system studied. In *Brassica napus* seeds both microsomes and oil body fractions have acyl-CoA elongation activity. The ATP-independent activity is found almost exclusively in the microsomal fraction (Fuhrmann et al., 1994), whereas the ATP-dependent activity is found in both microsomes and oil body fractions (Whitfield et al., 1993; Hlousek-Radojcic et al., 1995; Imai et al., 1995). Oil bodies prepared by Suc gradients showed minimal levels of the ER marker enzyme cholinephosphotransferase (Whitfield et al , 1993),

The exact nature of the endogenous primer has not been carefully studied and its identity has lately been questioned in both animal (Cinti et al., 1992) and plant systems (Evenson and Post-Beittenmiller, 1995; Hlousek-Radojcic et al., 1995). Microsomal preparations from plants and animals will synthesize very-long-chain fatty acids in the presence of ATP and malonyl-CoA from endogenous primer pools, exogenous free fatty acids, or acyl-CoAs. Specifically, elongation of fatty acids has been reported in microsomes isolated from pea (Bolton and Harwood, 1977), leek (Evenson and Post-Beittenmiller, 1995), and animals (see Cinti et al., 1992, and refs. therein). This elongase activity has been attributed to the activation of the supplied fatty acids (or endogenous fatty acids in which no primer is supplied) to acyl-CoAs by an endogenous ACS.

ACS is an ATP-dependent enzyme that has activity that has been reported in microsomes prepared from leek epidermis (Lessire and Cassagne, 1979), oil seeds (Ichihara et al., 1993), and rat liver (Guchait et al., 1966). Thus, the ATP requirement for endogenous acyl elongation and elongation of supplied fatty acids has been cited as evidence for ACS involvement (Nugteren, 1965). However, ACS activity is also CoA dependent, and under standard acyl elongase assay conditions no CoA is supplied. If CoA is not supplied to safflower (Ichihara et al., 1993) or leek microsomes (Lessire and Cassagne, 1979), no ACS activity is detected. Therefore, the endogenous CoA level is apparently insufficient for ACS activity under standard elongase assay conditions. Similarly, ACS activity in rat liver microsomes does not correlate with acyl elongase activity rates in the absence of exogenously supplied CoA, fatty acid, and Mg^{2+} (Cinti et al., 1992). Nugteren (1965) suggested that small amounts of CoA may be present in malonyl-CoA preparations, which would presumably be sufficient to support ACS activity under elongase assay conditions. However, this hypothesis had not been tested before the studies reported here. To elucidate the relationship of ACS activity and fatty acid elongation, we examined both ACS and elongase activities in the same preparations of leek microsomes or *B. napus* oil bodies under conditions in which maximum rates of fatty acid elongation were achieved.

MATERIALS AND METHODS

Substrates and Reagents

[2-^{14}C]Malonyl-CoA was synthesized according to Roughan (1994), using [2-^{14}C]acetate (54 mCi/mmol, DuPont NEN) and pea chloroplasts. [1-^{14}C]Stearic acid (18:0) (58 mCi/mmol) and [1-^{14}C]oleic acid (18:1) (50 mCi/mmol) were purchased from Amersham. [1-^{14}C]Stearoyl-CoA and [1-^{14}C]oleoyl-CoA were synthesized according to Taylor et al. (1990) with modifications for [1-^{14}C]stearoyl-CoA as previously described (Evenson and Post-Beittenmiller, 1995). Boron trifluoride (in 10% methanol) was from Alltech Associated, Inc. (Deerfield, IL). *Pseudomonas* ACS and all other chemicals were from Sigma.

Plant Material, Enzyme Assays, and Product Analyses

Leek (*Allium porrum* L.) microsomes were isolated from epidermis of rapidly expanding leaf and assayed for acyl elongation activity as previously described (Evenson and Post-Beittenmiller, 1995). Oil bodies were prepared from frozen developing *Brassica napus* L. cv Reston seeds and assayed for acyl elongation activity as described previously (Hlousek-Radojcic et al., 1995). After saponification, fatty acids were methylated with methanolic boron trifluoride and separated on KC_{18} RPTLC plates (Whatman) developed in acetonitrile:methanol:water (65:35:0.5, v/v). Quantification of radioactivity was carried out using a PhosphorImager and Image Quant (Molecular Dynamics, Sunnyvale, CA) or by scraping the radioactive bands and direct liquid scintillation counting of the silica gel.

All enzyme assays were conducted between two and four times with essentially the same qualitative results. The results of a single experiment are shown in each figure. Because of variations among microsomal or oil body preparations, mean values were not reported. ACS activity was assayed according to Groot et al. (1974) with the following modifications: the 25-μL assay mixture contained 80 mM Hepes-KOH, pH 7.2, 1 mM ATP, 125 mM $MgCl_2$, 0.5 mM NADPH, and 15 μM [1-^{14}C]oleate (NH_4^+ salt) with *B. napus* oil bodies or [1-^{14}C]stearate (NH_4^+ salt) with leek microsomes. Oleate and stearate were used because monounsaturated and saturated C-18 primers are the preferred substrates for *B. napus* oil bodies and leek microsome elongases, respectively. The concentrations of CoA used were as indicated in "Results" or in figure legends, and whenever indicated, 100 μM malonyl-CoA or 100 μM acetyl-CoA was added. Assays (2 and 30 min) were started with the addition of *B. napus* oil bodies (2–3 μg of protein) or leek microsomes (24–38 μg of protein), and stopped with an equal volume of 100 mM acetic acid and 4 volumes of water. Unreacted fatty acids were removed by four successive extractions into 800 μL of diethyl ether. The diethyl ether remaining on the surface of the aqueous phase was removed under a stream of nitrogen for 3 min. Acyl-CoAs and polar lipids were then extracted into *n*-butanol (3 × 100 μL). The butanol phases were pooled and the volume was reduced under a stream of nitrogen. Acyl-CoA and polar lipids were separated on Silica Gel 60 A plates (Whatman) by first developing plates to the full length in chloroform:methanol:glacial acetic acid:water (85:15:10:3, v/v) and then developing to one-third of the length in *n*-butanol:glacial acetic acid:water (5:2:3, v/v). Quantification of radioactivity was carried out as described above.

Acyl chain lengths of acyl-CoAs, PCs, and free fatty acid fractions were analyzed by RPTLC. Briefly, acyl-CoAs and PCs were eluted from scraped silica samples with *n*-butanol:glacial acetic acid:water (5:2:3, v/v), and free fatty acids were eluted with chloroform:methanol (2:1, v/v). Solvent volumes were reduced under nitrogen and lipids were transmethylated according to the procedure described by Sattler et al. (1996). Fatty acid methyl esters were prepared from the lipid fractions, separated by RPTLC, and quantified as described above.

Malonyl-CoA and [1-^{14}C]malonyl-CoA were purified (>99.8% CoA free) by HPLC (HP 1100, Hewlett-Packard) equipped with a diode array detector set to 260 nm, using a reverse-phase column (Microsorb-MV C18, Rainin, Woburn, MA) at a flow rate of 8 mL/min with an isocratic elution using 18% acetonitrile in 50 mM KPO_4 buffer, pH 5.2, for 20 min. Under these conditions, malonyl-CoA eluted at 9 min and CoASH eluted at 11 min. The column was then washed and reequilibrated by increasing the acetonitrile from 3 to 30% in 50 mM KPO_4 buffer, pH 5.2, over 1 min, followed by a 5-min wash with 30% acetonitrile, 50 mM KPO_4 buffer, pH 5.2, and finally returned to 3% acetonitrile, 50 mM KPO_4 over 1 min and held for an additional 14 min. Fractions that contained malonyl-CoA were collected, pooled, diluted 1:10 with water, and desalted on SepPak C_{18} minicartridges. Briefly, SepPak cartridges were prepared by washing (5 mL each) with decreasing concentrations of methanol (100, 75, 50, and 25%, v/v), followed by 1 mL of water, and, finally, 5 mL of 10 mM acetic acid. Samples were loaded by gravity and the procedure was carried out at 4°C. The column was washed with 5 mL of 10 mM acetic acid followed by 1 mL of water. Malonyl-CoA was eluted with 20% acetonitrile in 1-mL fractions. Peak fractions were identified either by radioactivity or by A_{260}, as applicable, and were combined and dried in a Speed-Vac (Savant Instruments, Farmingdale, NY). Dried malonyl-CoA was dissolved in water (adjusted to pH 3.0 with acetic acid) to 8 to 10 mM and stored at −20°C until used. Malonyl-CoA solutions remained free of CoA for at least 1 month when prepared and stored in this manner. The purity of the malonyl-CoA was analyzed using a reverse-phase C_{18} column at a flow rate of 1 mL/min and the following solvent system: a 5-min isocratic elution with 3% acetonitrile in 50 mM KPO_4 buffer, pH 5.2, followed by a 40-min gradient from 3 to 30% acetonitrile. Under these conditions malonyl-CoA eluted at 10.3 min and CoASH eluted at 11.9 min.

RESULTS AND DISCUSSION

B. napus Oil Bodies Will Effectively Elongate Fatty Acids in the Absence of CoA

We reported previously that leek microsomes elongate fatty acids at least as well as acyl-CoAs (Evenson and Post-Beittenmiller, 1995). Other researchers have reported similar findings in microsomes prepared from oil seeds (Bolton and Harwood, 1977) and rat hepatocyte microsomes (Nugteren, 1965). In *B. napus* the highest specific activities for acyl-CoA elongation are found in oil bodies (Imai et al., 1995). Therefore, oil body preparations were used to evaluate the relationship of ACS and fatty acid elongation activities and their CoA dependence. To ascertain if *B. napus* oil bodies would elongate fatty acids similarly to leek microsomes, the rates of [1-^{14}C]18:1 and [1-^{14}C]18:1-CoA elongation were compared (Fig. 1). We found that *B. napus* oil bodies used the fatty acid primer, in the presence of ATP, at higher rates than the acyl-CoA primer.

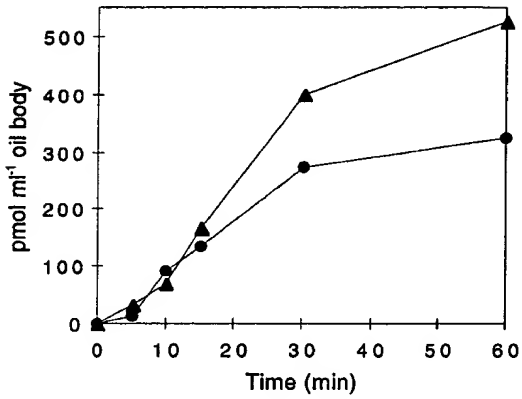

Figure 1. Comparison of fatty acid and acyl-CoA primers for acyl elongation in *B. napus* oil bodies. Either 15 μM [1-^{14}C]18:1 (▲) or 15 μM [1-^{14}C]18:1-CoA (●) was provided as the primer in acyl elongation assays. At the indicated times, assays were stopped and methyl esters were prepared from the saponified fatty acids. The elongated products were separated from the starting substrates by RPTLC and the radioactivity was quantified using a PhosphorImager and Image Quant (Molecular Dynamics).

ACS and Fatty Acid Elongation Activities

ACS activity in microsomes from rapidly expanding leek leaf epidermis and oil bodies from *B. napus* developing seeds were assayed by the method of Groot et al. (1974). Because we were interested in determining if ACS was contributing to fatty acid elongase activity, the incubation conditions used in these assays were essentially those used for elongase assays; i.e. the buffer, and the concentrations of the cofactors in common were the same as for elongase assays. ACS assays performed according to Lessire and Cassagne (1979) gave essentially the same results. In each case, ACS activity was detected in the microsomal membrane or oil body preparations. In the presence of ATP, CoA (50 μM), and ^{14}C-fatty acid, ACS activities were detected in both leek microsomes and in *B. napus* oil bodies. The rate reported for safflower microsomes (2520 nmol h^{-1} mg^{-1} protein) by Ichihara et al. (1993) is 2.5-fold higher than the rate reported here for *B. napus* oil bodies (962 nmol h^{-1} mg^{-1} protein), and the rate reported for leek microsomes (24 nmol h^{-1} mg^{-1} protein) by Lessire and Cassagne (1979) is 3-fold higher than our rate of 8.2 nmol h^{-1} mg^{-1} protein for leek microsomes. In both of these studies, the levels of CoA (0.5–1 mM) were at least 10-fold higher and the levels of fatty acid (0.5–0.8 mM) were more than 30-fold higher than the levels used in our studies.

To assess the CoA requirements for ACS and acyl elongation activities, a series of CoA concentrations, from 0 to 50 μM, was used with leek microsomes and *B. napus* oil bodies. As shown in Figure 2, in the absence of CoASH, ACS activities were very low in both the leek microsomes and the *B. napus* oil bodies. In leek microsomes (Fig. 2, left), ACS activity increased 38-fold with increasing concentrations of CoASH, indicating a clear dependence of ACS activity on supplied CoA. In contrast, elongase activity in the same preparations was unaffected by increasing CoA concentrations. Fatty acid elongation rates were similar

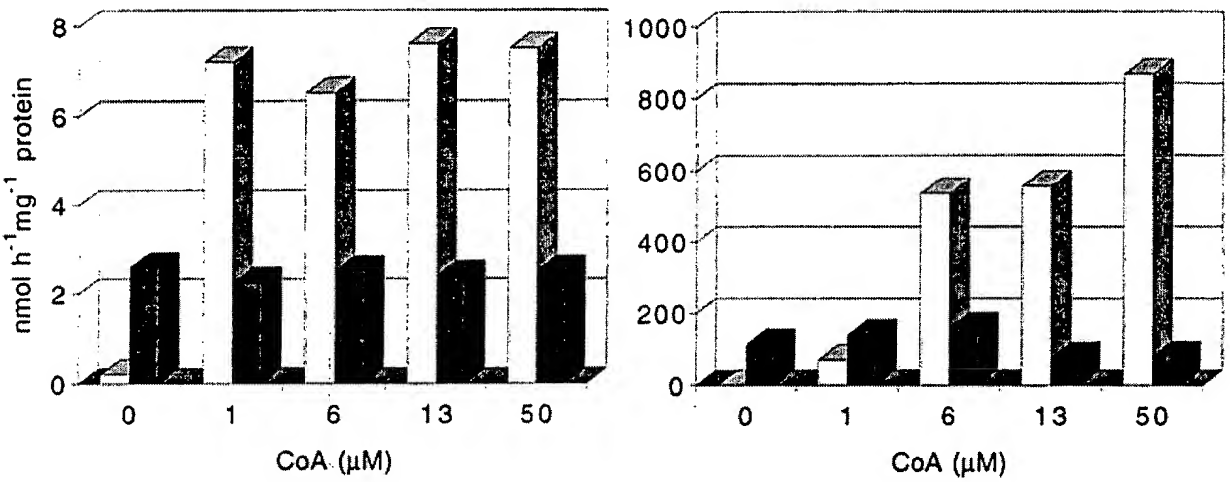

Figure 2. Effect of increasing CoA concentrations on fatty acid elongation and ACS activities in leek microsomes (left) and *B. napus* oil bodies (right). Standard ACS assays (open bars), using [1-^{14}C]18:0 with leek microsomes and [1-^{14}C]18:1 with *B. napus* oil bodies, or elongase assays (solid bars), using [1-^{14}C]18:0 with leek microsomes and [2-^{14}C]malonyl-CoA with *B. napus* oil bodies, were carried out with increasing CoA concentrations. Acyl-CoAs and fatty acid methyl esters were prepared and analyzed as described in "Materials and Methods" and the legend to Figure 1.

(2.2–2.6 nmol h^{-1} mg^{-1} protein) from 0 to 50 μM CoA. In *B. napus* oil bodies, ACS activity likewise was dependent on supplied CoA, whereas elongase activity was largely unaffected by CoA (Fig. 2, right). These data together indicated that microsomal and oil body preparations did not contain sufficient levels of CoA to support ACS activity, yet they supported high rates of fatty acid elongation. Furthermore, in *B. napus* oil bodies, ACS activity was 3- to 11-fold higher than fatty acid elongation activity when CoA was supplied at greater than 3 μM. Therefore, if ACS provides substrate for acyl elongation, a stimulation of acyl elongation would be expected unless the level of primer was saturating. However, the concentration of [1-^{14}C]18:0 (15 μM) was below saturating levels of the primer (Evenson and Post-Beittenmiller, 1995); therefore, we concluded that fatty acid elongation rates should have responded to increasing synthesis of acyl-CoAs if acyl-CoAs rather than fatty acids were a more direct substrate. Thus, the results clearly indicated that elongation rates were unaffected by increased ACS activities.

HPLC Purification of Malonyl-CoA

Commercially available malonyl-CoA and solutions of malonyl-CoA (if improperly prepared or stored) may contain small amounts of nonesterified CoA. Because 100 μM malonyl-CoA is routinely used in fatty acid elongation assays, even 1% contaminating CoASH could introduce significant levels of CoA. This could potentially provide the CoA necessary to synthesize acyl-CoAs from fatty acids in microsomal or oil body preparations during an elongase assay. To assess whether our malonyl-CoA solutions contained significant levels of CoASH or oxidized CoA, malonyl-CoA was analyzed by reverse-phase HPLC and quantified by A_{260}. We detected 1 to 3% CoA contamination in various lots of commercially available malonyl-CoA to provide 1 to 3 μM of CoA in the elongase assay. Therefore, we purified malonyl-CoA on a C_{18} reverse-phase column to >99.8% (based on the detection limits of the HPLC system; Fig. 3, top), and used this CoA-free malonyl-CoA for further studies of fatty acid elongation and acyl-CoA synthesis. We estimate that the purified fractions contained less than 0.2% nonesterified CoA.

CoA-Free Malonyl-CoA Supported Fatty Acid Elongation

Commercial preparations of malonyl-CoA had been used for the fatty acid elongation assays described above and in previous studies (Evenson and Post-Beittenmiller, 1995). We repeated the fatty acid elongation assays using the HPLC-purified malonyl-CoA and compared its effectiveness with a commercial preparation of malonyl-CoA and with our purified malonyl-CoA, which had been supplemented with 3 μM CoA (Fig. 4). It was evident that at the earliest time points the amount of elongated product did not differ significantly under the three treatments. In fact, the HPLC-purified malonyl-CoA was slightly more efficient than either the CoA-supplemented or the commercial malonyl-CoA preparations at generating greater levels of product at the later time points. These results indicated that contaminating CoA in the commercial preparations could not explain elongation of endogenously activated free fatty acids in the absence of supplied CoA.

We also considered whether hydrolysis of malonyl-CoA during the elongation reaction could provide sufficient CoA for activation of fatty acids. Such hydrolysis would presumably be independent of the purity of the malonyl-CoA. However, the data presented here (Fig. 4) appear inconsistent with malonyl-CoA hydrolysis being a source of CoA, unless this hydrolysis was very rapid. At 1 and 3 min the extent of elongation with CoA-free malonyl-CoA was equal to or greater than the extent with commercial or

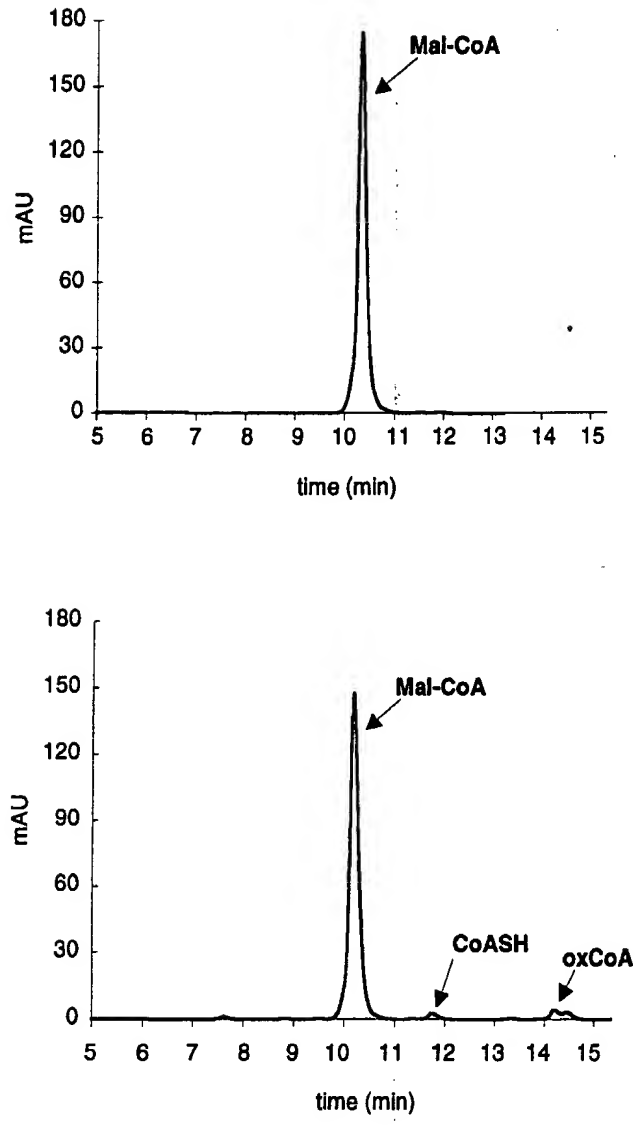

Figure 3. Essentially pure malonyl-CoA (>99.8%) was obtained from commercial preparations of malonyl-CoA after separation by HPLC chromatography using a C_{18} reverse-phase column (top). Commercial preparations of malonyl-CoA were shown to contain 1 to 3% CoASH and smaller amounts of oxidized CoA (bottom). mAU, Milliabsorbance unit.

CoA-dependent elongation, malonyl-CoA hydrolysis would need to occur at rates greater than 2 to 3% per min to provide adequate CoA for the earliest time points. To evaluate the possibility of rapid hydrolysis of malonyl-CoA under elongase assay conditions, we examined the fatty acid elongation reactions for the presence of CoA and loss of malonyl-CoA at different time periods. Using HPLC analysis, we were unable to detect any CoASH, oxidized CoA, or rapid loss of malonyl-CoA. We cannot rule out the possibility that any hydrolyzed CoA was rapidly reesterified to a fatty acid, although this is improbable because the levels required for significant ACS activity (Fig. 2) would be easily detected. Thus, if hydrolysis occurred during the elongase assay, it was insufficient to contribute substan-

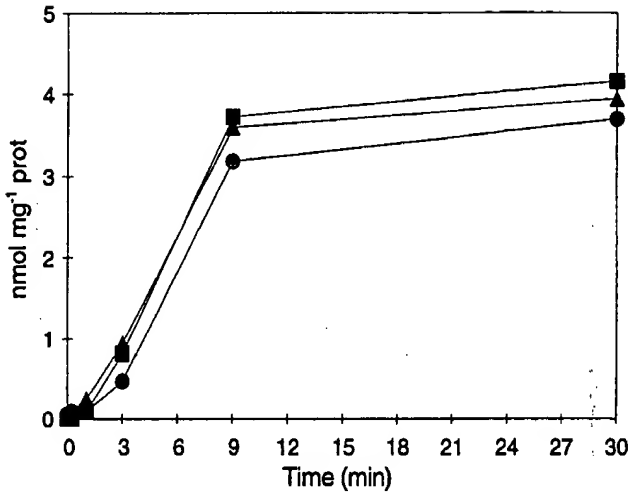

Figure 4. Comparison of fatty acid elongation activities in leek microsomes using [1-^{14}C]18:0 as the acyl primer and HPLC-purified malonyl-CoA (■), commercial malonyl-CoA (▲), or HPLC-purified malonyl-CoA supplemented with 3 μM CoASH (●). Assays were carried out and elongated products analyzed as described in Figure 1. prot, Protein.

18:0-CoA Was Synthesized during Elongation Reactions

Surprisingly, [1-^{14}C]18:0-CoA was produced in leek microsomes in the absence of CoA but in the presence of malonyl-CoA, as shown by analysis of the acyl chain lengths. Because the formation of [1-^{14}C]18:0-CoA occurred in the absence of CoA, it was unlikely to result from the action of ACS. Therefore, these data suggested that [1-^{14}C]18:0-CoA was formed by an esterification of the [^{14}C]18:0 fatty acid with malonyl-CoA. To examine in more detail the effect of malonyl-CoA on the formation of [1-^{14}C]18:0-CoA, we evaluated the CoA dependence of [1-^{14}C]18:0-CoA synthesis in the presence or absence of malonyl-CoA with increasing concentrations of CoA (Fig. 5). In the absence of malonyl-CoA, 18:0-CoA synthesis

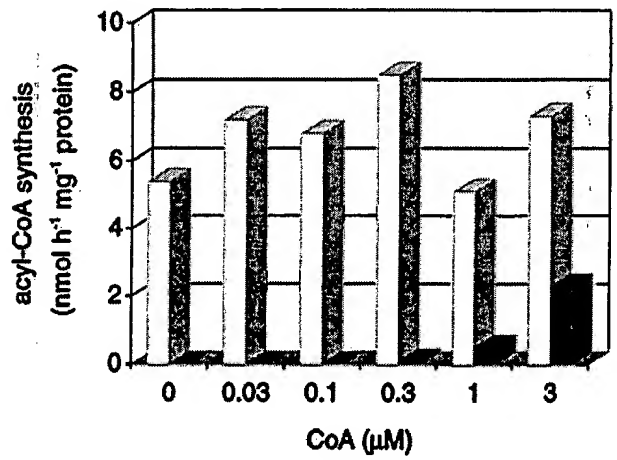

Figure 5. Effect of increasing CoA concentrations on [1-^{14}C]18:0-CoA formation in the presence (open bars) or absence (solid bars) of HPLC-purified malonyl-CoA in leek microsomes. Fatty acid elongation assays were carried out in the presence (100 μM) or absence

increased in response to increasing CoA concentrations, consistent with the presence of ACS activity. However, in the presence of CoA-free malonyl-CoA, even though substantial amounts of [1-^{14}C]18:0-CoA were synthesized, increasing CoA concentrations had no effect on acyl-CoA synthesis, indicating that an activity other than ACS was responsible for 18:0-CoA synthesis in leek microsomes.

The CoA-independent synthesis of 18:0-CoA observed in the presence of malonyl-CoA was not expected, and to our knowledge, it has not been previously reported. Consequently, we examined the cofactor requirements for this reaction using leek epidermal microsomes to determine if this synthesis was ATP dependent and if acetyl-CoA could substitute for malonyl-CoA (Fig. 6). Acyl-CoA synthesis occurred with both acetyl-CoA and malonyl-CoA, but only in the presence of ATP. The ATP requirement and the involvement of a free fatty acid suggest that the acyl-CoA synthesis was not a simple acyl-exchange reaction. Both the malonyl-CoA and the acetyl-CoA were analyzed by HPLC and were essentially CoA free (data not shown). Thus, the acyl-CoA synthesis could not be attributed to a large contamination of CoA, which is what would be required because this activity was 1.5 to 3.6 times greater than ACS activity with 3 μM CoA.

Does 18:0-CoA Synthesis Correlate with Fatty Acid Elongation?

Although 18:0-CoA was synthesized in the presence of malonyl-CoA under elongation conditions, we did not know if the synthesis of 18:0-CoA preceded or was necessary for fatty acid elongation. To address this issue, we examined the rate of appearance of [1-^{14}C]18:0 in [1-^{14}C]18:0-CoA and lipids and compared that with the rate of appearance of elongated fatty acids in these fractions. Elongase assays were carried out with CoA-free malonyl-CoA under standard conditions, and the acyl chain length composition of the acyl-CoA and lipid fractions was analyzed as described in "Materials and Methods." It is clear that [1-^{14}C]18:0 appeared in the PC fraction as rapidly as it appeared in the acyl-CoA fraction (Fig. 7). During the first 3 min we observed no difference in the accumulation of elongation products (20:0, 22:0, 24:0, and 26:0) between the PC and acyl-CoA fractions. At 9 min, after the reaction had progressed substantially, we did observe a somewhat higher level of elongated products in the acyl-CoA fraction, the significance of which is unclear. The appearance of radioactivity in the free fatty acids was not as rapid, suggesting a low level of hydrolysis (data not shown). This labeling pattern is similar to the pattern seen with 18:1-CoA and PC for oleate desaturase activity (Roughan, 1975; Stymne and Appelqvist, 1978; Slack et al., 1979).

As has been well documented, acyl groups are rapidly exchanged from acyl-CoAs into PC (Stymne and Glad, 1981; Stymne and Stobart, 1984; Griffiths et al., 1988a, 1988b). Because of this rapid exchange, acyl-CoAs can be

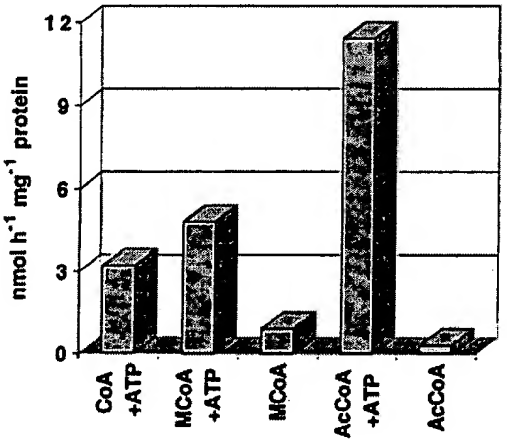

Figure 6. Effect of malonyl-CoA (MCoA), acetyl-CoA (AcCoA), ATP, and CoA on acyl-CoA synthesis in leek microsomes. Assays were carried out under standard elongase assay conditions using [1-^{14}C]18:0 and indicated cofactors (100 μM acetyl-CoA, 100 μM malonyl-CoA, 1 mM ATP, 3 μM CoA). The lipids were extracted and separated by TLC and the radioactive acyl-CoA and PC bands were quantified using a PhosphorImager and Image Quant (Molecular

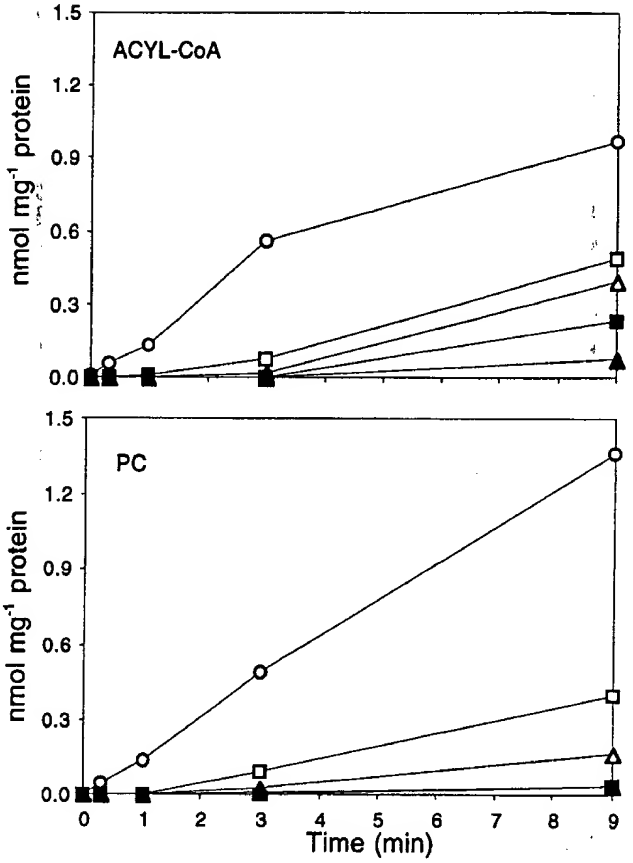

Figure 7. Progress curve using leek microsomes showing the appearance of ^{14}C in acyl moieties of acyl-CoAs and PC. Fatty acid elongation assays were carried out using [1-^{14}C]18:0 and stopped at the indicated times. The lipids were extracted and separated by TLC. The radioactive acyl-CoA and PC bands were scraped and the lipids were eluted, then saponified, methylated, separated by RPTLC, and quantified as described in "Materials and Methods" and in Figure 1. ○, [1-^{14}C]18:0; □, [1-^{14}C]20:0; △, [1-^{14}C]22:0; ■, [1-^{14}C]24:0; ▲, [1-^{14}C]26:0.

though the in vivo substrate is PC (Slack et al., 1979). Although we have not demonstrated this to be the case for acyl elongation, the data so far are consistent with this hypothesis. Similarly, the wax synthase activity, which esterifies fatty alcohols to a fatty acyl moiety, can use acyl-CoAs, but the in vivo substrate may be a phospholipid (Kolattukudy, 1967). The idea that PC or another glycerolipid is the source of acyl primer is an attractive hypothesis for the following reasons. First, acyl-CoAs are unlikely substrates for acyl elongation (Hlousek-Radojcic et al., 1995; this work). Second, PC as a major ER membrane component has accessibility to the elongase enzyme and provides a medium for solubilizing the hydrophobic growing acyl chain. Third, PC is a pool for desaturation precursors and therefore functions as a reservoir for some acyl chain modifications.

CONCLUSIONS

Several lines of evidence have led us to question whether acyl-CoAs are the immediate substrates for acyl elongation. First, rates of acyl elongation measured by [1-^{14}C]malonyl-CoA incorporation are 2.5-fold higher than the rates of elongation measured by either ^{14}C-fatty acid or [^{14}C]acyl-CoA incorporation, suggesting that a significant endogenous primer pool is present (Hlousek-Radojcic et al., 1995). Second, the specific activity of the supplied [^{14}C]acyl-CoA substrate is considerably higher than the specific activity of the synthesized elongated product in *B. napus* oil bodies. This implies that the product acyl-CoA is derived from a large endogenous pool. Measurements indicate that the 18:1-CoA pool in *B. napus* oil body preparations is less than 0.6 μM, which is at least 20-fold less than the concentration of [^{14}C]acyl-CoA supplied, and therefore, cannot be the pool that dilutes the supplied substrate. Information on the size of fatty acid pools in plants has not been reported to date, but free fatty acid levels in animal microsomes are reported to be 0.03 μmol mg^{-1} protein (Cinti et al., 1992, and refs. therein). Third, nonesterified fatty acids are elongated at higher rates and to greater levels than acyl-CoAs (Fig. 1) (Evenson and Post-Beittenmiller, 1995). Fourth, the elongation of fatty acids is accomplished in the absence of added CoA (Fig. 4) (Evenson and Post-Beittenmiller, 1995). Finally, preincubation studies with acyl-CoAs show that although the acyl-CoA pool is reduced by >95%, the subsequent acyl elongation activity is reduced only 2- to 3-fold (Evenson and Post-Beittenmiller, 1995; Hlousek-Radojcic et al., 1995).

In this study we provide evidence that ACS activity in the absence of supplied CoA cannot account for the ATP-dependent fatty acid elongation in leek microsomes and *B. napus* oil bodies. Furthermore, although acyl-CoAs were synthesized in vitro under conditions that support high rates of acyl elongation, the formation of acyl-CoAs was ATP and malonyl-CoA dependent, and it did not correlate with fatty acid elongation activity. Thus, although exogenous acyl-CoAs are readily elongated in *B. napus* and leek, we propose that ATP plays a role in the elongation process

In conclusion, we have shown that fatty acid elongation in leek microsomes and *B. napus* oil bodies was CoA independent and that CoA-free malonyl-CoA preparations were able to support high rates of fatty acid elongation. Therefore, ACS activity did not play a direct role in providing primer for acyl elongation in plants. Furthermore, we described an activity present in plant microsomes that synthesized acyl-CoA from [1-^{14}C]18:0 in the presence of malonyl-CoA or acetyl-CoA and that was dependent on ATP. In addition, [1-^{14}C]18:0 appeared in the PC and acyl-CoA fractions simultaneously with the appearance of the elongated products, which suggests that PC or other glycerolipid is a likely primer for acyl elongation.

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